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Effect of posttranscriptional regulatory elements on transgene expression and virus production in the context of retrovirus vectors

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Abstract

Ineffective transgene expression in a sufficient amount of target cells is still a limitation in retroviral vector mediated gene therapy. Thus, we systematically evaluated four genetic modulators, (i) the woodchuck posttranscriptional regulatory element (WPRE), (ii) the mouse RNA transport element (RTE), (iii) the constitutive transport element (CTE) of the simian retrovirus type 1 (SRV-1), and (iv) the 5′ untranslated region of the human heat shock protein 70 (Hsp70 5′UTR), all of them involved in the posttranscriptional control of mRNA nucleo/cytoplasmatic transport, RNA stability, and translation efficiency, in an MLV-based retrovirus vector context. Insertion of the WPRE into the retrovirus vector resulted in enhancement of transgene expression (EGFP) both in transfected virus producing cells as well as in infected recipient cells irrespective of the location in the vector. The best effect was observed with two copies of the WPRE, 3′ of the transgene and in the 3′ untranslated region of the vector backbone. However, oligomerization of this element does not further increase transgene expression. Presence of the WPRE resulted also in an increase in virus production. Introduction of the CTE and/or RTE in the retroviral vector did not alter transgene expression and infectious particle production. Positive effects were observed only in vectors harboring the CTE and/or RTE in combination with the WPRE. The activity of the Hsp70 5′UTR as a translational enhancer was found to be negligible in the context of the retroviral vector. However, interference of the Hsp70 5′UTR strong secondary structure with the packaging sequence of the viral RNA was experimentally excluded as being the cause of this. These data suggest that only the WPRE is a suitable element for the improvement of transgene expression and oncoretroviral vector production.

Keywords: Retrovirus vector; RNA transport element (RTE); CTE; WPRE; Hsp70

Introduction

Retroviral vectors are the most widely used delivery vehicles for human gene therapy (http://www.wiley.co.uk/genmed/clinical). However, the inability to achieve therapeutic levels of transgene expression in sufficient numbers of specific target cells still remains the obligatory problem (Greco et al., 2002). To overcome this obstacle, modulation of the recombinant mRNA at the posttranscriptional level has become an important consideration in the design of gene therapy vectors in general.

Most mRNAs are spliced and polyadenylated prior to their export from the nucleus to the cytoplasm (Hastings and Krainer, 2001; Weis, 2002). However, for the retroviral life cycle, the presence of non-spliced forms of the viral RNA in the cytoplasm is indispensable as well. To that end, many retroviruses have developed transport mechanisms to achieve this goal. In complex retroviruses, *trans*-acting proteins, such

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as the HIV Rev protein, facilitate export of unspliced and single spliced RNA. *Cis*-acting sequences called constitutive transport elements (CTE) were identified in several simple retroviruses, including Mason-Pfizer monkey virus (MPMV), simian retrovirus type D (SRV-1), Rous sarcoma virus (RSV), and endogenous murine retroviruses (Bray et al., 1994; Zolothukin et al., 1994; Paca et al., 2000; Wodrich et al., 2001). CTEs interact directly with cellular factors such as the tip-associated protein (Tap) and cause export of unspliced RNAs (Gruter et al., 1998).

Woodchuck hepatitis virus (WHV) harbors a posttranscriptional regulatory element (WPRE, Donello et al., 1998) that facilitates nucleocytoplasmic transport of RNA mediated by several alternative pathways that may be cooperative (Popa et al., 2002). In addition, the WPRE has been shown to act on additional posttranscriptional mechanisms to stimulate expression of heterologous cDNAs (Zufferey et al., 1999).

Sequences involved in the transport of mRNAs from the nucleus to cytoplasm have also been identified in several cellular genes (Ainger et al., 1997; Huang and Carmichael, 1997; Bassel et al., 1999). Most recently, using a Rev- and RRE-mutant of an HIV-1 proviral clone, a mouse posttranscriptional regulatory element involved in RNA cytoplasmic transport (RTE) has been identified (Nappi et al., 2001).

Regions flanking the coding sequences in eukaryotic mRNA molecules also contain elements involved in regulation of mRNA stability, transport, subcellular localization, and translation efficiency (Conne et al., 2000; Pesole et al., 2001; Wilkie et al., 2003). Specific regions capable of enhancing translation of the downstream genes were identified in the 5′ untranslated regions (UTR) from the vascular endothelial growth factor (VEGF) gene and the human heat shock protein 70 (Hsp70) gene (Huez et al., 1998; Vivinus et al., 2001).

Some of these *cis*-acting elements have been already used in current viral vector systems to achieve more potent transgene expression in the target cells. Enhanced transgene expression in adenovirus vectors, adeno-associated virus vectors, lentivirus vectors, and MLV-derived vectors harboring WPRE has been reported (Loeb et al., 1999; Mautino and Morgan, 2002; Ketteler et al., 2002; Xu et al., 2003). Constitutive transport elements of MPMV or SRV-1 have been exploited in order to improve titer and safety of lentivirus and MLV vectors (Mautino et al., 2000; Wodrich et al., 2000; Zhao et al., 2000).

In the present study, we describe the systematic evaluation of four different genetic elements, qualified for the regulation of expression both at the posttranscriptional and translational level, in the context of an oncoretroviral vector. A set of MLV-based retroviral vectors expressing the enhanced green fluorescent protein gene (EGFP) were progressively modified to include modulators of the nuclear/cytoplasmatic RNA transport (WPRE, CTE, RTE) as well as a translation enhancer element (Hsp70 5′UTR). To achieve the best impact on transgene expression, these elements were inserted either alone or in combination at various locations in the viral vector. Based on the obtained data, none of the alternative transport elements tested led to a reasonable increase in vector performance in terms of expression of the vector-inserted transgene except the WPRE. Thus, the data presented here are of importance for other researchers with respect to the choice of the most suitable element for the improvement of oncoretroviral vectors.

Results and discussion

WPRE facilitated enhancement of transgene expression and viral titer is position and copy number independent

The use of heterologous tissue-specific promoters inserted into the U3 region of the MLV 3′ long terminal repeat (LTR) allows tissue-specific expression of the transferred gene in promoter conversion (ProCon) vectors (Mrochen et al., 1997; Saller et al., 1998; Öztürk-Winder et al., 2002). Unfortunately, the replacement of elements within the long terminal repeats (LTRs) of retroviral vectors to achieve such transcriptional targeting is often associated with decreases in both titer and transgene expression (Hlavaty et al., 2004a, 2004b). Strategies exploiting the insertion of *cis*-acting elements like the WPRE which is known to facilitate nucleocytoplasmic transport and thereby increase transgene expression, have been employed to overcome these drawbacks in oncoretroviral, and more frequently in lentiviral vectors (Zufferey et al., 1999; Ketteler et al., 2002; Brun et al., 2003). In nearly all cases, the WPRE element was inserted downstream of the actual transgene as a single copy element.

In this study, we analyzed whether the WPRE mediated improvement in transgene expression and vector production is dependent on the site of insertion and/or the number of inserted elements in a retroviral vector carrying two transgenes. A set of ProCon vectors carrying 2 different heterologous promoters in the U3 region of the 3′LTR was constructed and used. A 600 bp fragment of the complete WPRE sequence, retaining 85% of its activity, was inserted into the retroviral vectors VEA, PCEmCMV, and PCEhCMV (Fig. 1; Donello et al., 1998). The impact of (i) the site of insertion – directly downstream of the EGFP transgene (Fig. 1, vectors VEWA, PCEWmCMV, and PCEWhCMV), in the 3′ untranslated region (3′UTR, Fig. 1, vectors PCEmCMVW and PCEhCMVW), and in both positions within the same vector (Fig. 1, vectors PCEWmCMVW and PCEWhCMVW) as well as (ii) the number of copies of the WPRE sequence introduced into the vector (Fig. 1, monomer – all abovementioned vectors, dimer inserted 3′ of the EGFP gene as in vectors VE2WA, PCE2WmCMV, and PCE2WhCMV, and a tetramer 3′ of the EGFP gene – vector VE4W) were analyzed both in transiently transfected 2GP19Talf packaging cells by

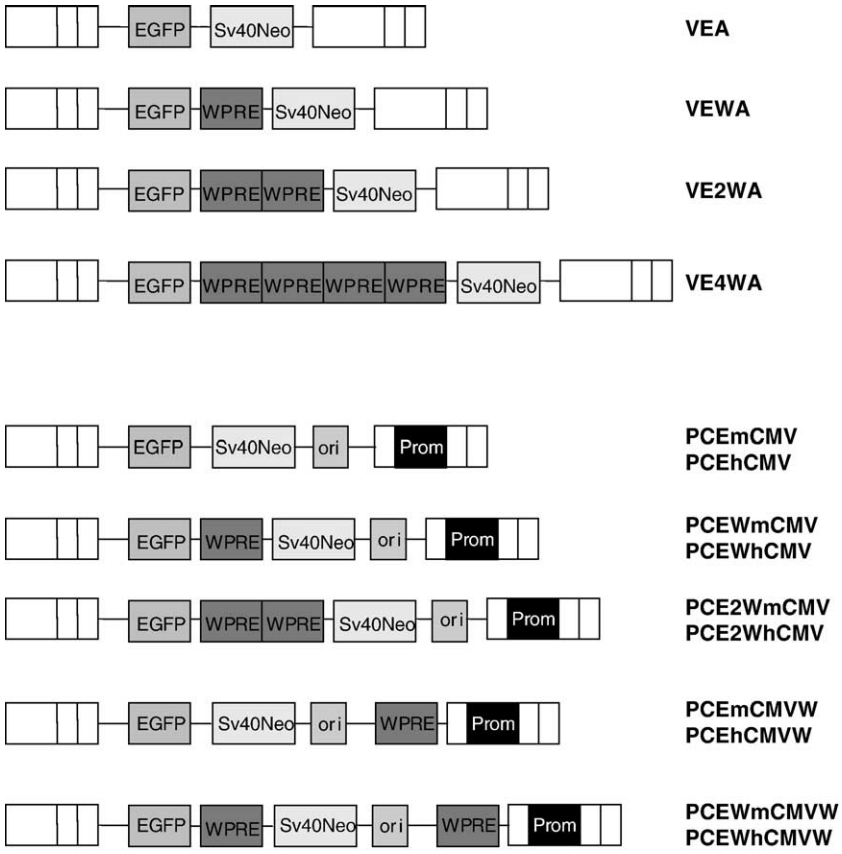

Fig. 1. Retroviral vector constructs. *VEA*—MLV-based vector (LTR consisting of Unique 3′ (U3), Repeated (R) and Unique 5′ (U5) regions) containing the enhanced green fluorescent protein gene (EGFP) as well as an internal SV40 promoter (SV40) controlling expression of the neomycin resistance gene (neo); *VEWA*—VEA with the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) in *sense* orientation 3′ of the EGFP gene; *VE2WA* and *VE4WA*—VEA with a dimer or tetramer of the WPRE in *sense* orientation 3′ of the EGFP gene; *PCEmCMV*, *PCEhCMV*—MLV-based ProCon vectors containing the EGFP gene as well as an internal SV40neo cassette and a hybrid 3′LTR with the murine (mCMV) or human (hCMV) CMV promoter cloned in the 3′LTR U3 region; *PCEWmCMV*, *PCEWhCMV*—PCEmCMV and PCEhCMV with the WPRE in *sense* orientation 3′ of the EGFP gene; *PCE2WmCMV*, *PCE2WhCMV*—PCEmCMV and PCEhCMV with a dimer of the WPRE in *sense* orientation 3′ of the EGFP gene; *PCEmCMVW*, *PCEhCMVW*—PCEmCMV and PCEhCMV with the WPRE in *sense* orientation cloned into the 3′ untranslated region (UTR) of the vector; *PCEWmCMVW*, *PCEWhCMVW*—PCEmCMV and PCEhCMV with two copies of the WPRE, 3′ of the EGFP gene as well as in the 3′UTR of the same vector, both in *sense* orientation.

evaluating transgene expression and vector production as well as in infected NIH3T3 cells by determining transgene expression (Fig. 1).

Generally, in the transfected virus producer cells, insertion of the WPRE into the basic ProCon vectors PCEmCMV and PCEhCMV resulted in an increase in transgene expression independent of the position of the WPRE within the vector and the WPRE copy number in all constructs. Introduction of a single WPRE (Fig. 1, vectors VEWA, PCEWmCMV, PCEWhCMV, PCEmCMVW, PCEhCMVW) led to a 2- to 3-fold increase in transgene expression, whereby the insertion of the WPRE 3′ of the EGFP gene (PCEWmCMV, PCEWhCMV) gave slightly better results compared to a WPRE location in the 3′UTR (PCEmCMVW, PCEhCMVW) (data not shown). Insertion of the WPRE monomer both 3′ of the EGFP gene and in the 3′UTR of the same vector (Fig. 1, PCEWmCMVW and PCEWhCMVW) led to a more than 5-fold increase in transgene expression as compared to PCEmCMV and PCEhCMV. Insertion of two and four copies of the WPRE (Fig. 1, vectors VE2WA, PCE2WmCMV, PCE2WhCMV, and vector VE4W), respectively, did not result in further increases in transgene expression. Similar effects with respect to the location and number of WPREs on transgene expression were also observed in infected NIH3T3 cells (data not shown). Based on these data, it seems that (i) the WPRE enhances transgene expression both in transfected packaging cells as well as in infected cells; (ii) insertion of the WPRE in the proximity of the transgene (3′ of the gene of interest) is superior to the insertion into the 3′ untranslated region of the retrovirus vector; (iii) the presence of more than one copy of the WPRE does not further enhance the transgene expression neither in transfected nor in infected cells except if monomers of the WPRE are inserted both 3′ of the transgene and into the 3′UTR of the same vector.

Consistent with the data obtained in transient transfection experiments, the WPRE improves transgene expression in stable virus producing cells (Table 1, compare line 1 vs. 2, 3 vs. 4/5, and 6 vs. 7/8). Surprisingly, in contrast to the data

Table 1
Evaluation of the WPRE effect on transgene expression, virus production, and virus titer

Virus vector[a]	MFI in transfected cells[b]	Real-time titer[c]	EGFP titer[d]	MFI in infected cells[e]
1 VEA	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0[f]	1.0 ± 0.0
2 VEWA	5.5 ± 1.4	1.4 ± 0.6	2.0 ± 1.5	2.7 ± 0.4
3 PCEmCMV	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
4 PCEWmCMV	4.4 ± 0.5	1.8 ± 0.2	2.2 ± 0.7	4.1 ± 0.5
5 PCEWmCMVW	5.0 ± 0.2	3.5 ± 0.4	2.1 ± 0.2	10.1 ± 0.6
6 PCEhCMV	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
7 PCEWhCMV	4.5 ± 0.2	2.1 ± 0.6	7.4 ± 1.6	1.7 ± 0.5
8 PCEWhCMVW	5.1 ± 0.1	2.8 ± 0.6	8.7 ± 0.4	2.3 ± 0.1

All values are calculated relative to the data – set as 1 – obtained with the corresponding vector lacking the WPRE.
[a] 2GP19Talf cells were stably transfected with a variety of vector plasmids.
[b] The expression of EGFP in the packaging cells was monitored by mean fluorescence intensity (MFI) measurement.
[c] The number of virion containing RNA in the supernatant of the packaging cells was evaluated by real-time RT-PCR (real-time titer).
[d] The efficiency of infection of NIH3T3 cells was measured by determination of the percentage of green cells via FACS analysis 48 h postinfection (EGFP titer).
[e] Expression of EGFP in the infected cells was monitored by MFI measurement.
[f] Average absolute infectious titer has been 1×10^6 gfu/ml.

obtained after transient vector transfection, no significant differences were observed between vectors PCEWmCMV vs. PCEWmCMVW and PCEWhCMV vs. PCEWhCMVW (Table 1, lines 4 vs. 5 and 7 vs. 8). Increased transgene expression in virus producing cells was accompanied by an increase in the number of packaged vector genomes in cell culture supernatants from the vector producing cells, in the amount of infective virus particles as well as in transgene expression in infected NIH3T3 cells (Table 1, compare line 1 vs. 2, 3 vs. 4/5 and 6 vs. 7/8). However, these observations are in contradiction to those from Zufferey et al. (1999) and Mautino and Morgan (2002), in which the presence of the WPRE did not affect vector titer and infectivity, respectively. This might be due to the fact that in our vector system the produced vector RNA is the limiting factor for virus production, and thus virus production will be enhanced by providing greater amounts of vector RNA facilitated by the activity of the WPRE.

In line with the results obtained in transient transfection experiments, the maximal positive effect on transgene expression and virus production as compared to corresponding vectors lacking WPRE was achieved using vectors containing monomers of the WPRE inserted 3′ of the gene of interest and in the 3′UTR of the same vector (PCEWmCMVW and PCEWhCMVW, respectively). Even though these vectors were superior to all other vectors tested, the potential risk of recombination between the two identical WPRE sequences inserted close to each other, has to be taken into consideration. Thus, viruses harboring only a WPRE monomer downstream of the transgene were used to analyze the WPRE effect in different recipient cells. Infection of mouse (PAN02) and human (293 HEK, PANC-1, MiaPaca-2, and Paca44) cell lines with vectors VEWA, PCEWmCMV, and PCEWhCMV revealed increased transgene expression up to 5-fold for VEWA, 4-fold for PCEWmCMV, and 3-fold for PCEWhCMV as compared to the respective vectors without the WPRE (VEA, PCEmCMV, PCEhCMV; Fig. 2). Consistent with published data, the actual level of increase of expression was dependent on the type of recipient cells used and the promoter selected to control transgene expression (Ramezani et al., 2000; Brun et al., 2003).

Neither the mouse RTE nor the viral CTE is able to enhance virus production and transgene expression in context of an MLV retroviral vector

In this study, the effects of the CTE and RTE RNA transport elements on transgene expression and vector production in context of the retroviral ProCon vector PCEmCMV were analyzed alone, in combination, or together with the WPRE. First, the impact of the site of insertion was evaluated in transient transfection/infection experiments. The CTE, RTE, or both elements together were selectively inserted into the 3′UTR of the retroviral vectors (Fig. 3, vectors PCEmCMVR, PCEmCMVC, and

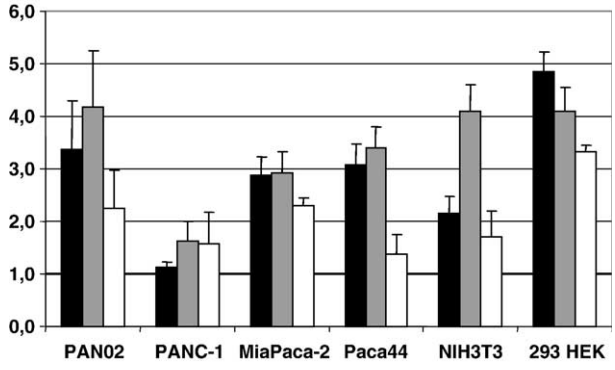

Fig. 2. Effect of the WPRE on transgene expression in infected cells. Different mouse and human cell lines were infected with viruses harboring the WPRE (VEWA—black column, PCEWmCMV—grey column, PCEWhCMV—white column) as well as with their parental counterparts lacking the WPRE (VEA, PCEmCMV, PCEhCMV). Forty-eight hours postinfection the expression of the EGFP was monitored by measuring the MFI. Relative transgene expression with respect to the transgene expression (relative MFI) in cells infected with the parental vectors VEA, PCEmCMV, and PCEhCMV (always set as an arbitrary unit equal to 1), respectively, is shown for each vector and cell line combination.

PCEmCMVRC), since (i) it is the position where the CTE is located in SRV1 and MPMV, (ii) the RTE is located in the 3′ region of the IAP retroelements, and (iii) it was reported that the CTE needs to be located at a certain distance to the poly(A) signal of the RNA to be functional (Bray et al., 1994; Zolothukin et al., 1994; Rizvi et al., 1997; Nappi et al., 2001). An insertion site immediately downstream of the EGFP transgene (Fig. 3, vectors PCERmCMV, PCERCmCMV) was chosen as a second site of insertion since in this case only proviral RNA produced from the promoter in the retrovirus 5′LTR is expected to be affected, while shorter RNA molecules transcribed from the internal SV40 promoter remain unaffected. This might be of importance in case the pool of proteins assisting in the cellular RNA transport machinery is limited. Transgene expression in 2GP19Talf cells producing these vectors as well as in infected mouse NIH3T3 and human HeLa cells did not reveal any significant increase in mean fluorescence intensity (MFI) of EGFP (data not shown). Interestingly, the combination of RTE and CTE actually appears to reduce transgene expression in infected mouse cells (NIH3T3) but not in infected human cells (HeLa, data not shown). This is different to the data obtained by Smulevitch et al. (submitted for publication) who showed a strong synergistic effect on RNA export when combining RTE and CTE in a lentiviral context. Surprisingly, constructs harboring RTE and/or CTE cloned in *antisense* orientation displayed slightly better

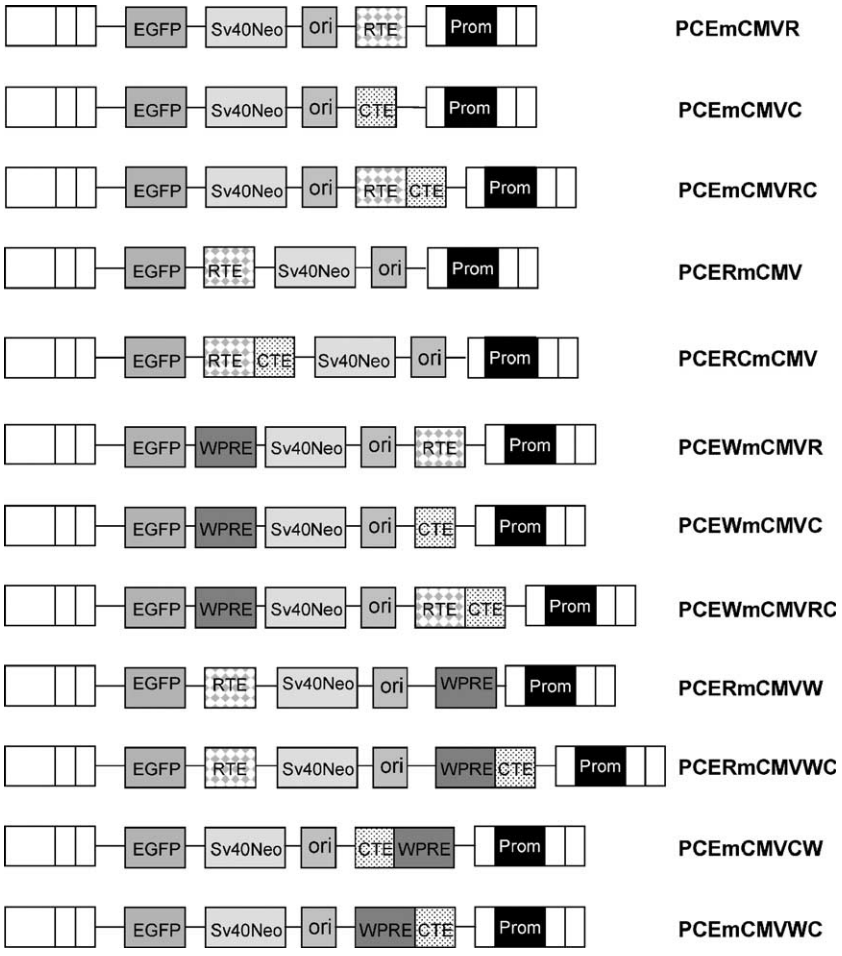

Fig. 3. Retroviral vector constructs harboring the RTE and/or CTE based on ProCon vector PCEmCMV. *PCEmCMVR*—PCEmCMV with the RTE in *sense* orientation cloned into the 3′UTR of the vector; *PCEmCMVC*—PCEmCMV carrying the CTE in *sense* orientation cloned into the 3′UTR of the vector; *PCEmCMVRC*—PCEmCMV with a combination of RTE and CTE in *sense* orientation cloned into the 3′UTR of the vector; *PCERmCMV*—PCEmCMV with the RTE in *sense* orientation 3′ of the EGFP gene; *PCERCmCMV*—PCEmCMV with a combination of RTE and CTE in *sense* orientation cloned 3′ of the EGFP gene; *PCEWmCMVR*—PCEmCMV with the WPRE in *sense* orientation 3′ of the EGFP gene and the RTE in *sense* orientation cloned into the 3′UTR of the same vector; *PCEWmCMVC*—PCEmCMV with the WPRE in *sense* orientation 3′ of the EGFP gene and the CTE in *sense* orientation cloned into the 3′UTR of the same vector; *PCEWmCMVRC*—PCEmCMV with the WPRE in *sense* orientation 3′ of the EGFP gene and RTE and CTE in *sense* orientation cloned into the 3′UTR of the same vector; *PCERmCMVW*—PCEmCMV with the RTE in *sense* orientation 3′ of the EGFP gene and WPRE in *sense* orientation cloned into the 3′UTR of the same vector; *PCERmCMVWC*—PCEmCMV with the RTE in *sense* orientation 3′ of the EGFP gene and a combination of WPRE and CTE in *sense* orientation cloned into the 3′UTR of the same vector; *PCEmCMVCW*—PCEmCMV with the CTE and WPRE both in *sense* orientation cloned into the 3′UTR of the same vector; and finally *PCEmCMVWC*—PCEmCMV with the WPRE and CTE both in *sense* orientation cloned into the 3′UTR of the same vector.

effects compared to corresponding constructs with the RTE and/or CTE cloned in *sense* orientation (data not shown). This is a clear contradiction to the RTE function in the context of a lentivirus, where RTE activity was only shown when inserted in *sense* orientation, whereas insertion in the opposite transcriptional orientation resulted in a failure to produce viruses (Nappi et al., 2001). These data reveal that the restriction in expression of MLV is distinct from that of lentiviruses and that different restrictions may apply to different mRNAs. Thus, individual elements within different RNAs studied may function differently, or may be inactive.

To see whether the WPRE mediated effect can be further potentiated by insertion of an alternative RNA transport element, vectors harboring the RTE, the CTE, or both in the 3′UTR in addition to the 3′ of the EGFP gene located WPRE (Fig. 3, vectors PCEWmCMVR, PCEWmCMVC, and PCEWmCMVRC), containing the RTE 3′ of the EGFP gene and the WPRE in the 3′UTR (Fig. 3, PCERmCMVW), or carrying a combination of the WPRE and the CTE in the 3′UTR with (Fig. 3, PCERmCMVWC) or without (Fig. 3, PCEmCMVWC, PCEmCMVCW) the RTE 3′ of the EGFP were constructed and analyzed. No major differences in transgene expression between vectors harboring WPRE alone and those carrying the WPRE and the RTE and/or CTE in any combination and position were observed in transiently and stable transfected virus producer cells (Table 2, MFI in producer cells) as well as in infected NIH3T3 and HeLa cells (Table 2, MFI in infected cells). Surprisingly, however, differences in MFI between infected mouse NIH3T3 and human HeLa cells were detected. No positive effect of the two transport elements on virus production was observed (Table 2, real-time titer, EGFP titer). A possible explanation for this, as well as for the lack of improvement in transgene expression, is that the major splice acceptor is not present in the ProCon vector as in most retroviral vectors. The CTE function is suspected to be an accelerator of export of unspliced RNA, at least in messages where splicing occurs, and is thereby shifting the balance in favor of the full length message instead of spliced transcripts (Schambach et al., 2000). Thus, it is unlikely that an increase of unspliced exported ProCon vector messages can be seen in our system, where splicing will not occur. A second explanation might be that the transport elements, which are known to be highly structured themselves, influence the overall secondary structure of the vector RNA thereby decreasing RNA stability or translation efficiency. This would, however, implicate that the WPRE acts not at the level of nucleocytoplasmic RNA export, but rather constitutes a translational enhancer, as has been previously described (Schambach et al., 2000).

To rule out the possibility that the obtained data are cell-specific and therefore not representative, we analyzed the effect of the RTE/CTE on transgene expression in various cell lines of mouse and human origin. Mouse PAN02 and NMuMG cells as well as human PANC-1, Paca44, MiaPaca-2, and BxPc-3 cells were infected with supernatants from cells stably producing the various retroviral vectors. In line with the previous observation, the effect of the RTE/CTE on transgene expression in infected mouse cells was rather negative for all constructs harboring the transport elements in *sense* orientation and negligible for constructs carrying them in *antisense* orientation as compared to the corresponding vector without the WPRE (data not shown). In the infected human cells, the RTE/CTE effect on transgene expression was negligible in either orientation (data not shown).

Table 2
Effects of the RTE and/or CTE in a retroviral vector on transgene expression, virus production, and virus titer

Virus vector[a]	MFI in producer cells[b]	Real-time titer[c]	EGFP titer[d] NIH3T3	MFI in infected cells[e]	
				NIH3T3	HeLa
1 PCEmCMV	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
2 PCEmCMVW	2.7 ± 0.1	3.0 ± 0.8	2.7 ± 0.1	2.2 ± 0.1	2.0 ± 0.4
3 PCEmCMVR	1.1 ± 0.1	0.8 ± 0.3	1.3 ± 0.3	0.6 ± 0.0	1.1 ± 0.5
4 PCEmCMVRasn	1.5 ± 0.1	2.4 ± 0.0	0.9 ± 0.2	1.3 ± 0.1	1.1 ± 0.5
5 PCEmCMVRC	1.1 ± 0.1	1.1 ± 0.4	1.1 ± 0.3	0.4 ± 0.1	1.1 ± 0.5
6 PCEmCMVRCasn	0.8 ± 0.1	2.0 ± 1.4	0.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.5
7 PCEmCMVC	1.0 ± 0.0	4.2 ± 2.2	0.3 ± 0.3	0.4 ± 0.1	0.9 ± 0.4
8 PCEmCMVCasn	1.4 ± 0.1	1.2 ± 0.7	0.7 ± 0.5	1.2 ± 0.1	1.1 ± 0.5
9 PCEWmCMV	4.4 ± 0.5	1.8 ± 0.2	2.2 ± 0.7	4.1 ± 0.5	3.9 ± 2.1
10 PCERmCMV	0.8 ± 0.0	2.6 ± 1.0	2.2 ± 0.7	0.4 ± 0.0	0.5 ± 0.2
11 PCERmCMVasn	1.0 ± 0.0	1.5 ± 0.7	1.5 ± 0.4	1.7 ± 0.2	1.1 ± 0.5
12 PCERCmCMV	1.5 ± 0.5	1.5 ± 0.5	0.4 ± 0.3	0.1 ± 0.0	0.7 ± 0.4
13 PCERCmCMVasn	2.2 ± 0.1	3.1 ± 0.9	1.2 ± 0.3	1.4 ± 0.1	1.5 ± 0.6

All values shown are relative to those obtained with the parental vector PCEmCMV.
[a] 2GP19Talf cells were stably transfected with a variety of vector plasmids.
[b] The expression of EGFP in the packaging cells was monitored by MFI measurement.
[c] The number of virion containing RNA in the supernatant of the packaging cells was evaluated by real-time RT-PCR (real-time titer).
[d] The efficiency of infection of NIH3T3 cells was measured by determining the percentage of green cells by FACS analysis 48 h postinfection (EGFP titer).
[e] Expression of EGFP in the infected cells was monitored by MFI measurement.

To verify whether the RTE, the CTE, or the combination of both elements used here can exert their described function as RNA transport elements, lentiviral constructs carrying a truncated HIV genome with a deleted rev/RRE system but with intact RNA export inhibiting gag gene sequences (pNLgag), and with the respective RTE/CTE elements (pNLgagM26, pNLgagCTE, pNLgagM26CTE) were tested for gag-mRNA export using an HIV-1 Gag-specific capture ELISA. Compared to the lentiviral construct pNLgag containing none of the RTE and/or CTE elements, a 12-fold higher Gag level was detected in cells transfected with the RTE/CTE containing vector pNLgagM26CTE (data not shown). A 5-fold increase was observed in cells transfected with the CTE only containing vector pNLgagCTE and a modest 1.5-fold increase in cells transfected with the RTE containing vector pNLgagM26 (data not shown). Even if the RTE alone does not seem to significantly increase the HIV-gag expression, in combination with the CTE, it shows a strong synergistic effect in a lentiviral construct which is in agreement with a previous observation of studying this combination of elements in poorly expressed lentiviral mRNAs (Smulevitch et al., submitted for publication), but as already shown above, not in a retroviral construct.

Based on these data, it seems that (i) neither the RTE nor the CTE enhances transgene expression in retroviral vector transfected packaging cells as well as in infected cells, (ii) thus, no position dependence of the RTE/CTE (insertion 3′ of the transgene vs. insertion into the 3′UTR of the vector) was detected, (iii) multimerization of the RTE or CTE does not enhance transgene expression, neither in transfected nor in infected cells, and (iv) the only positive effect on transgene expression is achieved when the RTE and/or CTE is/are present in combination with the WPRE within the same vector. However, this effect is similar to that achieved with virus harboring the WPRE only and thus seems to be solely dependent on the activity of the WPRE.

Effect of the Hsp70 5′UTR on transgene expression and virus production

To study the effect of the Hsp70 5′UTR on translation in a retroviral context, we inserted the 218 bp long Hsp70 5′UTR fragment immediately upstream of the ATG codon of the EGFP gene into vectors VEA, VEWA, PCEmCMV, and PCEWmCMV and transfected the resulting plasmids pVUEA, pVUEWA, pPCUEmCMV, and pPCUEWmCMV (Fig. 4) stably into 2GP19Talf packaging cells. No increase in transgene expression and virus production from these cells was observed when compared to stable virus producers carrying the corresponding vectors VEA, VEWA, PCEmCMV, and PCEWmCMV (Table 3, MFI in producer cells). NIH3T3 cells infected with the respective viruses as well revealed no enhanced EGFP synthesis (Table 3, MFI in infected cells). Since the Hsp70 5′UTR sequence which is known to form strong secondary structures is inserted in close proximity to the extended packaging signal which also forms a strong secondary structure, one might expect interaction or sterical inhibition of these elements leading to a decrease in the function of one or both sequences, i.e. a reduced ability of the proviral RNA to be packaged. However, real-time RT-PCR analyses of virus supernatant revealed no reduction in the amount of infectious virus particles produced from cells carrying vectors with the Hsp70 5′UTR compared to cells producing Hsp70 5′UTR deficient vectors (Table 3, compare real-time titer and EGFP titer of vectors with and without the Hsp70 5′UTR).

To exclude the possibility that the secondary structure of the packaging signal is somehow negatively influencing the action of the Hsp70 5′UTR as a translational enhancer, eukaryotic expression constructs harboring EGFP gene with (pCOX-2UEGFP) and without (pCOX-2EGFP) the Hsp70 5′UTR under the control of cyclooxygenase-2 promoter were transfected into human 293 HEK and PANC-1 cells. Interestingly, consistent with the data obtained in NIH3T3 cells infected with the retroviral Hsp70 5′UTR-constructs,

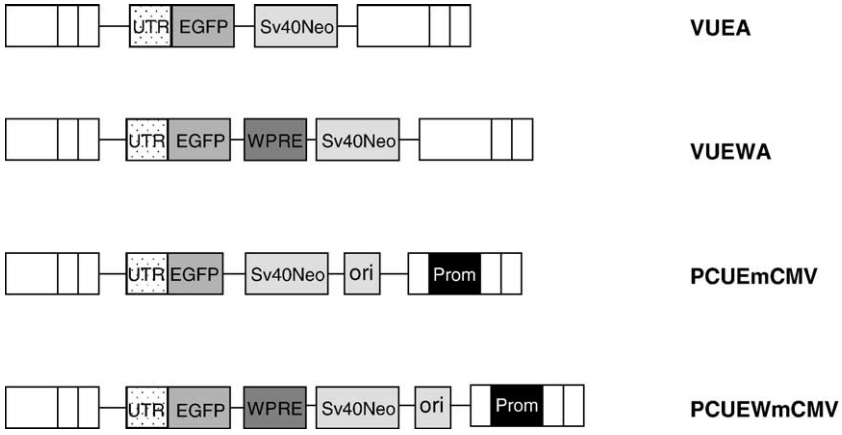

Fig. 4. Retroviral vector constructs harboring the human Hsp70 5′UTR. *VUEA*—VEA with the Hsp70 5′UTR cloned immediately upstream of the EGFP gene; *VUEA*—VUEA containing the WPRE in *sense* orientation 3′ of the EGFP gene; *PCUEmCMV*—PCEmCMV with the Hsp70 5′UTR cloned immediately upstream of the EGFP gene; *PCUEWmCMV*—PCUEmCMV carrying the WPRE in *sense* orientation 3′ of the EGFP gene.

Table 3
Effects of the Hsp70 5′UTR in context of a retroviral vector on transgene expression, virus production, and virus titer

Virus vector[a]	MFI in producer cells[b]	Real-time titer[c]	EGFP titer[d]	MFI in infected cells[e]
1 VEA	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
2 VUEA	2.4 ± 0.1	0.9 ± 0.3	0.9 ± 0.1	1.4 ± 0.1
3 VEWA	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
4 VUEWA	1.4 ± 0.1	1.2 ± 0.5	1.0 ± 0.1	1.6 ± 0.1
5 PCEmCMV	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
6 PCUEmCMV	1.3 ± 0.1	1.3 ± 0.2	1.5 ± 0.3	1.4 ± 0.1
7 PCEWmCMV	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
8 PCUEWmCMV	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.4 ± 0.1

Values shown are calculated relative to the corresponding vectors lacking the Hsp70 5′UTR.
[a] 2GP19Talf cells were stably transfected with a variety of vector plasmids.
[b] The expression of EGFP in the packaging cells was monitored by MFI measurement.
[c] The number of virion containing RNA in the supernatant of the packaging cells was evaluated by real-time RT-PCR (real-time titer).
[d] The efficiency of infection of NIH3T3 cells was measured by determining the percentage of green cells by FACS analysis 48 h postinfection (EGFP titer).
[e] Expression of EGFP in the infected cells was monitored by MFI measurement.

but, in contrast to data by Vivinus et al. (2001), who observed an up to 10-fold increase in gene expression, an only slight, non-significant increase in EGFP synthesis was observed (data not shown).

In this light, it should be mentioned that much of the published data regarding the role of 5′ untranslated regions as translational enhancers are controversial. Stein et al. (1998), for example, obtained a 40-fold increase in secreted alkaline phoshatase (SEAP) protein expression from a monocistronic expression vector harboring a 163bp long SP163 element, derived from the 5′UTR of the vascular endothelial growth factor (VEGF). The activity of this IRES like element was shown to be independent of the cell type used. In contrast, Johansen et al. (2003) recently described a reduction in SEAP protein levels in rat cells transfected with constructs containing the SP163 translational enhancer.

Previously, the presence of an IRES in the leader sequence in the different strains of the murine leukemia virus as well as in the mouse VL30 retrotransposon was described (Vagner et al., 1995; Berlioz and Darlix, 1995; Lopez-Lastra et al., 1999). This sequence co-locates with the elongated packaging signal which is also present in the ProCon vectors. Very recently, an internal ribosome entry site (IRES) with a similar activity to the classical picornavirus IRES elements has been discovered within the Hsp70 5′UTR sequence (Rubtsova et al., 2003). In spite of these data, it seems unlikely that an additional benefit on transgene expression can be obtained from linking two IRES sequences due to sterical hindrance, a theory which would be supported by our data.

In summary, our studies demonstrate that among the four posttranscriptionally acting elements (WPRE, RTE, CTE, and Hsp70 5′UTR) analyzed here, only the insertion of the WPRE led to a reasonable increase in vector performance in terms of expression of the vector-inserted transgene. Thus, these data may be helpful for construction of optimized and improved retrovirus vectors for gene therapy harboring the WPRE but also for saving efforts on further evaluation of the less favorable elements.

Materials and methods

Plasmid construction

The DNA sequence containing a fragment of the WPRE (GenBank accession no. J02442, pos. 1086–1694) was amplified by PCR from plasmid pSPWAS using specific primers with *Not*I and *Cla*I restriction sites, respectively, and subsequently cloned into plasmid pCR2.1 (Invitrogen). The resulting plasmids were named (in accordance to the restriction site in the primer) pWPRE CC, pWPRE NC, pWPRE CN, and pWPRE NN. To construct retrovirus vectors VEWA, PCEWmCMV, and PCEWhCMV, the WPRE was released from plasmid pWPRE NN by digestion with *Not*I and inserted into the appropriate vector plasmids pVEA, pPCEmCMV, and pPCEhCMV, previously linearized with *Not*I (Hlavaty et al., 2004a, 2004b). To generate retrovirus vectors with a dimer of the WPRE 3′ of the EGFP gene, plasmids pWPRE NC and pWPRE CN were digested with *Not*I and *Cla*I. Subsequently, the WPRE-containing fragments were ligated to *Not*I-linearized plasmids pVEWA, pPCEWmCMV, and pPCEWhCMV in a three fragment ligation. The resulting constructs were named pVE2WA, pPCE2WmCMV, and pPCE2WhCMV. Additionally, the vector VE4WA, harboring four copies of the WPRE (two dimers), was cloned using the same strategy as described above. To obtain vectors pPCEmCMVW, pPCEhCMVW, pPCEWmCMVW, and pPCEWhCMVW, which carry a copy of the WPRE in the 3′ untranslated region (UTR) of the retrovirus vectors, the 600 bp *Cla*I fragment of pWPRE CC was inserted into plasmids pPCEmCMV, pPCEhCMV, pPCEWmCMV, and pPCEWhCMV linearized with *Cla*I.

An up-regulatory mutant of the mouse RNA transport element RTEm26 (further indicated as RTE), the simian retrovirus type D constitutive transport element (CTE), and a cassette containing both RTE and CTE were PCR amplified from plasmid pNLgagM26CTE (Smulevitch et al., 2005) using specific primers with restriction site

extensions for *Not*I and *Cla*I and cloned into plasmid pCR2.1. The resulting plasmids were named in accordance with the insert and the restriction sites in the primers as pRTE NN, pRTE NC, pRTE CC, pRTE CN pCTE CC, pCTE CN, pCTE NC, pRTECTE NN, and pRTECTE CC. For all cloning strategies used in this study, T4 DNA polymerase was used to convert sticky ends to blunt ends. To obtain plasmid pPCEmCMVR, the blunted *Eco*RI fragment containing the full length RTE from pRTE NN was ligated to the *Cla*I-linearized and blunted vector pPCEmCMV. Similarly, the RTE-CTE-containing *Eco*RI fragment of plasmid pRTECTE NN was treated with T4 DNA polymerase and ligated to the *Cla*I-linearized, T4 DNA polymerase treated vector pPCEmCMV giving rise to vector PCEmCMVRC. The CTE released from plasmid pCTE CC by *Cla*I digestion was inserted into the *Cla*I-linearized vector pPCEmCMV to generate plasmid pPCEmCMVC. *Age*I–*Csp*45I DNA fragments containing the EGFP gene and the SV40neo cassette of plasmids pPCEmCMVR, pPCEmCMVC, and pPCEmCMVRC were replaced by the *Age*I–*Csp*45I fragment of PCEWmCMV containing the EGFP, WPRE, and SV40neo sequences. The resulting constructs were named PCEWmCMVR, PCEWmCMVC, and PCEWmCMVRC. To clone vectors pPCERmCMV and pPCERmCMVW, the *Not*I-released RTE fragment of plasmid pRTE NN was ligated to the *Not*I-linearized vectors pPCEmCMV and pPCEmCMVW. Similarly, the *Not*I-released RTE-CTE fragment from pRTECTE NN ligated to *Not*I-linearized plasmid pPCEmCMV yielded pPCERCmCMV plasmid. Vector pPCEmCMVCW was cloned by triple ligation of the CTE fragment released from plasmid pCTE CN by *Cla*I–*Not*I double digestion, the WPRE fragment released from plasmid pWPRE NC by *Not*I and *Cla*I digestion, and the *Cla*I-linearized plasmid pPCEmCMV. Similarly, pPCEmCMVWC was created by ligation of the WPRE fragment from plasmid pWPRE CN (released with *Cla*I and *Not*I), the CTE fragment from plasmid pCTE CN (released with *Not*I and *Cla*I), and the *Cla*I-linearized plasmid pPCEmCMV. The *Age*I–*Csp*45I fragment of pPCEmCMVWC containing the viral LTRs was ligated with the EGFP-containing *Age*I–*Csp*45I fragment of pPCERmCMV to yield pPCERmCMVWC. Plasmid pNLgagM26 was obtained from plasmid pNLgagM26CTE by *Xba*I–*Sal*I excision of the CTE followed by self-ligation of the T4 DNA polymerase treated backbone, whereas plasmid pNLgagCTE was constructed by removing the RTE element after digestion with *Cla*I. To create pNLgag, a *Sac*II–*Sal*I digestion of plasmid pNLgagM26CTE was used to remove the RTE and CTE elements, the backbone was treated with T4 DNA polymerase and self-ligated.

Genomic DNA from 293 human embryonal kidney cells (293 HEK) was isolated using a DNeasy Tissue Kit (Qiagen) according to the manufacturer's recommendation. The Hsp70 5′UTR (GenBank accession no. M11717, pos. 276–488) was PCR amplified using specific primers carrying recognition sites for *Eco*RI (forward primer) and *Nco*I (reverse primer) and the genomic DNA from 293 HEK cells as template. Amplified fragments were subsequently cloned into plasmid pCR2.1 giving rise to plasmid pHsp70UTR. The identity of the cloned insert was confirmed by sequencing. A 218 bp *Eco*RI–*Nco*I fragment of pHsp70UTR was inserted into the 4510 bp *Eco*RI–*Nco*I fragment of pCMLVU3 creating plasmid pCMLVU3H-sp70UTR (Hlavaty et al., 2004b). To clone pVUEA and pVUEWA, the 1051 bp *Bsr*GI fragment of pCMLVU3H-sp70UTR was ligated to the 5835 bp *Bsr*GI fragment of pVEA and the 6444 bp *Bsr*GI fragment of VEWA, respectively. To produce pPCUEmCMV, the 1969 bp *Asc*I–*Not*I fragment of pCMLVU3Hsp70UTR was ligated with the 7286 bp *Asc*I–*Not*I fragment of pPCEmCMV. A 609 bp *Not*I fragment from pWPRE NN was inserted into pPCUEmCMV linearized with *Not*I to create plasmid pPCUEWmCMV.

A short 400 bp fragment of the cyclooxygenase-2 promoter (GenBank accession no. AF276953, pos. 863–1260) was amplified from plasmid pCOXsd2EGFP (kindly provided by Dr. Holzmüller) using specific primers and subcloned into plasmid pCR2.1 to create plasmids pCR2.1COX-2s sen and pCR2.1COX-2s asn. The promoter-containing *Eco*RV–*Hind*III fragment of pCR2.1COX-2s asn was ligated to the *Nru*I–*Hind*III backbone fragment of pCMVEGFP (Walter et al., 2000). The resulting construct pCOX-2EGFP was digested with *Spe*I and *Nco*I and ligated to the *Nco*I–*Spe*I fragment of plasmid pHsp70UTR harboring the Hsp70 5′UTR sequence. The final plasmid was named pCOX-2UEGFP.

Cell culture and FACS analysis

Human 2GP19Talf amphotropic retroviral packaging cells (Pambalk et al., 2002), 293 HEK cells (ATCC CRL-1573), HeLa cells (ATCC CCL-2), HeLa cells stably expressing the HIV-1 tat gene (HLtat, Felber et al., 1990), PANC-1 (ATCC CRL-1469), Paca-44 (Löhr et al., 1994), MiaPaca-2 (ATCC CRL-1420), PAN02 (Corbett et al., 1984), and NMuMG (ATCC CRL-1636) were grown in Dulbecco's modified Eagle's medium (DMEM/Glutamax; Life Technologies) supplemented with 10% foetal calf serum (FCS, Life Technologies). NIH3T3 cells (ATCC CRL-1658) were maintained in DMEM/Glutamax/5% FCS (Owens, 1974) and BxPc-3 cells (ATCC CRL-1687) in RPMI medium supplemented with 10% FCS. Transfection, infection, and analysis of cells via FACS were performed as described elsewhere (Hlavaty et al., 2004b).

RNA extraction and real-time RT-PCR

Viral RNA extraction from cell culture supernatants and real-time RT-PCR was performed as described previously (Hlavaty et al., 2004a).

HIV-1 gag ELISA test

The HIV-1 p24 Antigen Capture Assay Kit (Biological Products Laboratory, Frederick Cancer Research and Development Center, Frederick, MD) was used to estimate the amount of the HIV-1 Gag protein in the samples. HLtat cells were transiently co-transfected with 0.5 μg of plasmid pCMV-dsRed-EXPRESS (Clontech) and 4.5 μg of gag-gene containing plasmids pNLgagM26CTE, pNLgagM26, pNLgagCTE, and pNLgag using the ExGen transfection reagent (MBI Fermentas) according to the manufacturer's recommendation. Forty-eight hours later, cells were harvested and lysed in 1% Triton X-100 (Sigma) by repeated freezing and thawing. Protein concentration was estimated using a Protein Dc Assay Kit (Bio-Rad). 10 μg of total protein extract per well was used for HIV-1 p24 ELISA detection according to the instructions provided by the manufacturer.

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References

Ainger, K., Avossa, D., Diana, A.S., Barry, C., Barbarese, E., Carson, J.H., 1997. Transport and localization elements in myelin basic protein mRNA. J. Cell. Biol. 123, 4895–4901.

Bassel, G.J., Oleynikov, Y., Singer, R.H., 1999. The travels of mRNAs through all cells large and small. FASEB J. 13, 447–454.

Berlioz, C., Darlix, J.L., 1995. An internal ribosomal entry mechanism promotes translation of murine leukemia virus gag polyprotein precursors. J. Virol. 69, 2214–2222.

Bray, M., Prasad, S., Dubay, J.W., Hunter, E., Jeang, K.T., Rekosh, D., Hammarskjold, M.L., 1994. A small element from the Mason-Pfizer monkey virus genome makes human immunodeficiency virus type 1 expression and replication Rev-independent. Proc. Natl. Acad. Sci. U.S.A. 91, 1256–1260.

Brun, S., Faucon-Biguet, N., Mallet, J., 2003. Optimization of transgene expression at the posttranscriptional level in neural cells: implication for gene therapy. Mol. Ther. 7, 782–789.

Conne, B., Stutz, A., Vassalli, J.D., 2000. The 3′ untranslated region of messenger RNA: a molecular 'hotspot' for pathology? Nat. Med. 6, 637–641.

Corbett, T.H., Roberts, B.J., Leopold, W.R., Peckham, J.C., Wilkoff, L.J., Griswold Jr, D.P., Schabel Jr., F.M., 1984. Induction and chemotherapeutic response of two transplantable ductal adenocarcinomas of the pancreas in C57BL/6 mice. Cancer Res. 44, 717–726.

Donello, J.E., Loeb, J.E., Hope, T.J., 1998. Woodchuck hepatitis virus contain a tripartite posttranscriptional regulatory element. J. Virol. 72, 5085–5092.

Felber, B.K., Drysdale, C.M., Pavlakis, G.N., 1990. Feedback regulation of human immunodeficiency virus type 1 expression by the Rev protein. J. Virol. 64, 3734–3741.

Greco, O., Scott, S.D., Marples, B., Dachs, G.U., 2002. Cancer gene therapy: 'delivery, delivery, delivery'. Front. Biosci. 7, 1516–1524.

Gruter, P., Tabernero, C., von Kobbe, C., Schmitt, C., Saavedra, C., Bachi, A., Wilm, M., Felber, B.K., Izaurralde, E., 1998. TAP, the human homolog of Mex67p, mediates CTE-dependent RNA export from the nucleus. Mol. Cell 5, 649–659.

Hastings, M.L., Krainer, A.R., 2001. Pre-mRNA splicing in the new millenium. Curr. Opin. Cell Biol. 13, 302–309.

Hlavaty, J., Stracke, A., Klein, D., Salmons, B., Günzburg, W.H., Renner, M., 2004a. Multiple modifications allow high-titer production of retroviral vectors carrying heterologous regulatory elements. J. Virol. 78, 1384–1392.

Hlavaty, J., Portsmouth, D., Stracke, A., Salmons, B., Günzburg, W.H., Renner, M., 2004b. Effects of sequences of prokaryotic origin on titer and transgene expression in retroviral vectors. Virology 330, 351–360.

Huang, Y., Carmichael, G.G., 1997. The mouse histone H2a gene contains a small element that facilitates cytoplasmic accumulation of intron-less gene transcripts and of unspliced HIV-1-related mRNAs. Proc. Natl. Acad. Sci. U.S.A. 94, 10104–10109.

Huez, I., Creancier, L., Audigier, S., Gensac, M.C., Prats, A.C., Prats, H., 1998. Two independent internal ribosome entry sites are involved in translation initiation of vascular endothelial growth factor mRNA. Mol. Cell. Biol. 18, 6178–6190.

Johansen, J., Tornøe, J., Møller, A., Johansen, T.E., 2003. Increased in vitro and in vivo transgene expression levels mediated through *cis*-acting elements. J. Gene Med. 5, 1080–1089.

Ketteler, R., Glaser, S., Sandra, O., Martens, U.M., Klingmüller, U., 2002. Enhanced transgene expression in primitive hematopoietic progenitor cells and empryonic stem cells efficiently transduced by optimized retroviral hybrid vectors. Gene Ther. 9, 477–487.

Loeb, J.E., Cordier, W., Harris, M.E., Weitzman, M.D., Hope, T.J., 1999. Enhanced expression of transgenes from adeno-associated virus vectors with the woodchuck hepatitis virus posttranscriptional regulatory element: implications for gene therapy. Hum. Gene Ther. 10, 2295–2305.

Lopez-Lastra, M., Ulrici, S., Gabus, C., Darlix, J.L., 1999. Identification of an internal ribosome entry segment in the 5′ region of the mouse VL30 retrotransposon and its use in the development of retroviral vectors. J. Virol. 73, 8393–8402.

Löhr, M., Trautmann, B., Gottler, M., Peters, S., Zauner, I., Maillet, B., Kloppel, G., 1994. Human ductal adenocarcinomas of the pancreas express extracellular matrix proteins. Br. J. Cancer 69, 144–151.

Mautino, M.R., Morgan, R.A., 2002. Enhanced inhibition of human immunodeficiency virus type 1 replication by novel lentiviral vectors expressing human immunodeficiency virus type 1 envelope antisense RNA. Hum. Gene Ther. 13, 1027–1037.

Mautino, M.R., Keiser, N., Morgan, R.A., 2000. Improved titers of HIV-based lentiviral vectors using the SRV-1 constitutive transport element. Gene Ther. 7, 1421–1424.

Mrochen, S., Klein, D., Nikol, S., Smith, J.R., Salmons, B., Günzburg, W.H., 1997. Inducible expression of $p21^{WAF-1/CIP-1/SDI-1}$ from a promoter conversion retroviral vector. J. Mol. Med. 75, 820–828.

Nappi, F., Schneider, R., Zolotukhin, A., Smulevitch, S., Michalowski, D., Bear, J., Felber, B.K., Pavlakis, G.N., 2001. Identification of a novel posttranscriptional regulatory element by using a *rev*- and RRE-mutated human immunodeficiency virus type 1 DNA proviral clone as a molecular trap. J. Virol. 75, 4558–4569.

Owens, R.B., 1974. Glandular epithelial cells from mice: a method for selective cultivation. J. Natl. Cancer Inst. 52, 1375–1378.

Öztürk-Winder, F., Renner, M., Klein, D., Müller, M., Salmons, B., Günzburg, W.H., 2002. The murine whey acidic protein promoter directs expression to human mammary tumors after retroviral transduction. Cancer Gene Ther. 9, 421–431.

Paca, R.E., Ogert, R.A., Hibbert, C.S., Izaurralde, E., Beemon, K.L., 2000. Rous sarcoma virus DR posttranscriptional elements use a novel RNA export pathway. J. Virol. 74, 9507–9514.

Pambalk, K., Hohenadl, C., Salmons, B., Günzburg, W.H., Renner, M., 2002. Specific packaging of spliced retroviral vector transcripts lacking the Ψ-region. Biochem. Biophys. Res. Commun. 293, 239–246.

Pesole, G., Mignone, F., Gissi, C., Grillo, G., Licciulli, F., Liuni, S., 2001. Structural and functional features of eukaryotic mRNA untranslated regions. Gene 276, 73–81.

Popa, I., Harris, M.E., Donello, J.E., Hope, T.J., 2002. CRM1-dependent function of a *cis*-acting RNA export element. Mol. Cell. Biol. 22, 2057–2067.

Ramezani, A., Hawley, T.S., Hawley, R.G., 2000. Lentiviral vectors for enhanced gene expression in human hematopoietic cells. Mol. Ther. 2, 458–469.

Rizvi, T.A., Schmidt, R.D., Lew, K.A., 1997. Mason-Pfizer monkey virus (MPMV) constitutive transport element (CTE) functions in a position-dependent manner. Virology 236, 118–129.

Rubtsova, M.P., Sizova, D., Dmitriev, S.E., Ivanov, D.S., Prassolov, V.S., Shatsky, I.N., 2003. Distinctive properties of the 5′-untranslated region of human Hsp70 mRNA. J. Biol. Chem. 278, 22350–22356.

Saller, R.M., Öztürk, F., Salmons, B., Günzburg, W.H., 1998. Construction and characterization of a hybrid mouse mammary tumor virus/murine leukemia virus-based retroviral vector. J. Virol. 72, 1699–1703.

Schambach, A., Wodrich, H., Hildinger, M., Bohne, J., Kräusslich, H.-G., Baum, C., 2000. Context dependence of different modules for posttranscriptional enhancement of gene expression from retroviral vectors. Mol. Ther. 2, 435–444.

Smulevitch, S., Michalowski, D., Zolotukhin, A.S., Schneider, R., Bear, J., Roth, P., Pavlakis, G.N., Felber, B.K., 2005. Structural and functional analysis of the RNA transport element, a member of an extensive family present in the mouse genome. J. Virol. 79, 2356–2365.

Smulevitch, S., Bear, J., Alicea, C., Gegerfelt, A.S., Michalowski, D., Rosati, M., Zolotukhin, A.S., Pavlakis, G.N., Felber, B.K., submitted for publication. Combination of two potent mRNA export elements, RTE and CTE, synergistically increases expression of unstable HIV and SIV mRNAs.

Stein, I., Itin, A., Einat, P., Skaliter, R., Grossman, Z., Keshet, E., 1998. Translation of vascular endothelial growth factor mRNA by internal ribosome entry: implication for translation under hypoxia. Mol. Cell. Biol. 18, 3112–3119.

Vagner, S., Waysbort, A., Marenda, M., Gensac, M.C., Amalric, F., Prats, A.C., 1995. Alternative translation initiation of the Moloney murine leukemia virus mRNA controlled by internal ribosome entry involving the p57/PTB splicing factor. J. Biol. Chem. 270, 20376–20383.

Vivinus, S., Baulande, S., van Zanten, M., Campbell, F., Topley, P., Ellis, J.H., Dessen, P., Coste, H., 2001. An element within the 5′ untranslated region of human *Hsp70* mRNA which acts as a general enhancer of mRNA translation. Eur. J. Biochem. 268, 1908–1917.

Walter, I., Fleischmann, M., Klein, D., Müller, M., Salmons, B., Günzburg, W.H., Renner, M., Gelbmann, W., 2000. Rapid and sensitive detection of enhanced green fluorescent protein expression in paraffin sections by confocal laser scanning microscopy. Histochem. J. 32, 99–103.

Weis, K., 2002. Nucleocytoplasmic transport: cargo trafficking across the border. Curr. Opin. Cell. Biol. 14, 328–335.

Wilkie, G.S., Dickson, K.S., Gray, N.K., 2003. Regulation of mRNA translation by 5′- and 3′-UTR-binding factors. TRENDS Biol. Sci. 28, 182–188.

Wodrich, H., Schambach, A., Kräusslich, H.G., 2000. Multiple copies of the Mason-Pfizer monkey virus constitutive transport element lead to enhanced HIV-1 gag expression in a context-dependent manner. Nucleic Acids Res. 28, 901–910.

Wodrich, H., Bohne, J., Gumz, E., Welker, R., Kräusslich, H.-G., 2001. A new RNA element located in the coding region of a murine endogenous retrovirus can functionally replace the Rev/Rev-responsive element system in human immunodeficiency virus type 1 gag expression. J. Virol. 75, 10670–10682.

Xu, Z.L., Mizuguchi, H., Mayumi, T., Hayakawa, T., 2003. Woodchuck hepatitis virus post-transcriptional regulation element enhances transgene expression from adenovirus vectors. Biochem. Biophys. Acta 1621, 266–271.

Zhao, Y., Low, W., Collins, M.K.L., 2000. Improved safety and titre of murine leukaemia virus (MLV)-based retroviral vectors. Gene Ther. 7, 300–305.

Zolothukin, A.S., Valentin, A., Pavlakis, G.N., Felber, B.K., 1994. Continuous propagation of RRE(-) and Rev(-)RRE(-) human immunodeficiency virus type 1 molecular clones containing a *cis*-acting element of simian retrovirus type 1 in human peripheral blood lymphocytes. J. Virol. 68, 7944–7952.

Zufferey, R., Donello, J.E., Trono, D., Hope, T., 1999. Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. J. Virol. 73, 2886–2892.